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ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.(54) Title: A PROCESS FOR THE PRODUCTION OF L-AMINO ACIDS USING STRAINS OF THE ENTEROBACTERIACEAE  
FAMILY WHICH CONTAIN AN ENHANCED FADR OR ICLR GENE

(57) Abstract: This invention relates to a process for the production of L-amino acids, in particular L-threonine, in which the following steps are performed: a) fermentation of microorganisms of the Enterobacteriaceae family which produce the desired L-amino acid, in which at least one or more of the genes, selected from the group iclR and fadR, or nucleotide sequences coding therefor, are enhanced, in particular overexpressed, b) accumulation of the desired L-amino acid in the medium or in the cells of the bacteria and c) isolation of the desired L-amino acid.

WO 03/038106 A2

**A Process for the Production of L-Amino Acids Using Strains  
of the Enterobacteriaceae Family which Contain an Enhanced  
fadR or iclR Gene**

Field of the Invention

5 The present invention relates to a process for the production of L-amino acids, in particular L-threonine, using strains of the Enterobacteriaceae family, in which at least one or more genes, selected from the group iclR and fadR, is/are enhanced.

10 Prior Art

L-Amino acids, in particular L-threonine, are used in human medicine and in the pharmaceuticals industry, in the food industry and very particularly in animal nutrition.

It is known that L-amino acids are produced by fermentation  
15 of strains of Enterobacteriaceae, in particular Escherichia coli (E. coli) and Serratia marcescens. Due to their great significance, efforts are constantly being made to improve the production process. Improvements to the process may relate to measures concerning fermentation technology, such  
20 as for example stirring and oxygen supply, or to the composition of the nutrient media, such as for example sugar concentration during fermentation, or to working up to yield the product by, for example, ion exchange chromatography, or to the intrinsic performance  
25 characteristics of the microorganism itself.

The performance characteristics of these microorganisms are improved using methods of mutagenesis, selection and mutant selection. In this manner, strains are obtained which are resistant to antimetabolites, such as for example the  
30 threonine analogue  $\alpha$ -amino- $\beta$ -hydroxyvaleric acid (AHV), or are auxotrophic for regulatorily significant metabolites and produce L-amino acids, such as for example L-threonine.

For some years, methods of recombinant DNA technology have likewise been used to improve strains of the Enterobacteriaceae family which produce L-amino acids by amplifying individual amino acid biosynthesis genes and  
5 investigating the effect on production.

#### Object of the Invention

The object of the invention is to provide novel measures for the improved fermentative production of L-amino acids, in particular L-threonine.

#### 10 Summary of the Invention

The invention provides a process for the fermentative production of L-amino acids, in particular L-threonine using microorganisms of the Enterobacteriaceae family, which in particular already produce L-amino acids and in  
15 which at least one or more of the nucleotide sequence(s) which code(s) for the genes *iclR* and *fadR* is/are enhanced.

#### Detailed Description of the Invention

Any subsequent mention of L-amino acids or amino acids should be taken to mean one or more amino acids, including  
20 the salts thereof, selected from the group comprising L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine.  
25 L-threonine is particularly preferred.

In this connection, the term "enhancement" describes the increase in the intracellular activity of one or more enzymes or proteins in a microorganism, which enzymes or proteins are coded by the corresponding DNA, for example by  
30 increasing the copy number of the gene or genes, by using a strong promoter or a gene or allele which codes for a

corresponding enzyme or protein having elevated activity and optionally by combining these measures.

The enhancement, in particular overexpression, measures increase the activity or concentration of the corresponding protein in general by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, at most by 1000% or 2000%, relative to the activity or concentration of the wild type protein, or the activity or concentration of the protein in the starting microorganism.

10 The process is characterized in that the following steps are performed:

- 15 a) fermentation of microorganisms of the Enterobacteriaceae family, in which one or more of the genes, selected from the group *iclR* and *fadR*, or nucleotide sequences coding therefor, are enhanced, in particular overexpressed,
- b) accumulation of the corresponding L-amino acid in the medium or in the cells of the microorganisms of the Enterobacteriaceae family and
- 20 c) isolation of the desired L-amino acid, wherein constituents of the fermentation broth and/or the biomass in their entirety or fractions thereof (>0 to 100%) optionally remain in the product.

The microorganisms provided by the present invention are 25 capable of producing L-amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, optionally starch, optionally cellulose or from glycerol and ethanol. They are representatives of the Enterobacteriaceae family selected from the genera *Escherichia*, *Erwinia*, *Providencia* and 30 *Serratia*. The genera *Escherichia* and *Serratia* are preferred. Within the genus *Escherichia*, the species *Escherichia coli* and *Escherichia coli* serotype O157:H7 are preferred. Within the genus *Serratia*, the species *Serratia marcescens*.

Suitable strains of the genus *Escherichia*, in particular L-threonine producing strains, in particular of the species *Escherichia coli* are for example

- Escherichia coli TF427
- 5 Escherichia coli H4578
- Escherichia coli KY10935
- Escherichia coli VNIIGenetika MG442
- Escherichia coli VNIIGenetika M1
- Escherichia coli VNIIGenetika 472T23
- 10 Escherichia coli BKIIM B-3996
- Escherichia coli kat 13
- Escherichia coli KCCM-10132.

Suitable L-threonine producing strains of the genus *Serratia*, in particular of the species *Serratia marcescens*  
15 are for example

- Serratia marcescens* HNr21
- Serratia marcescens* TLR156
- Serratia marcescens* T2000.

L-Threonine producing strains from the Enterobacteriaceae  
20 family preferably have, inter alia, one or more of the genetic or phenotypic features selected from the group:  
resistance to  $\alpha$ -amino- $\beta$ -hydroxyvaleric acid, resistance to thialysine, resistance to ethionine, resistance to  $\alpha$ -methylserine, resistance to diaminosuccinic acid,  
25 resistance to  $\alpha$ -aminobutyric acid, resistance to borrelidin, resistance to rifampicin, resistance to valine analogues such as for example valine hydroxamate, resistance to purine analogues, such as for example 6-dimethylaminopurine, dependency on L-methionine,  
30 optionally partial and offsettable dependency on L-isoleucine, dependency on meso-diaminopimelic acid, auxotrophy with regard to threonine-containing dipeptides, resistance to L-threonine, resistance to L-homoserine, resistance to L-lysine, resistance to L-methionine,

resistance to L-glutamic acid, resistance to L-aspartate,  
resistance to L-leucine, resistance to L-phenylalanine,  
resistance to L-serine, resistance to L-cysteine,  
resistance to L-valine, sensitivity to fluoropyruvate,  
5 defective threonine dehydrogenase, optional ability to  
utilize sucrose, enhancement of the threonine operon,  
enhancement of homoserine dehydrogenase I-aspartate kinase  
I, preferably the feedback-resistant form, enhancement of  
homoserine kinase, enhancement of threonine synthase,  
10 enhancement of aspartate kinase, optionally the feedback-  
resistant form, enhancement of aspartate semialdehyde  
dehydrogenase, enhancement of phosphoenolpyruvate  
carboxylase, optionally the feedback-resistant form,  
enhancement of phosphoenolpyruvate synthase, enhancement of  
15 transhydrogenase, enhancement of the RhtB gene product,  
enhancement of the RhtC gene product, enhancement of YfiK  
gene product, enhancement of a pyruvate carboxylase, and  
attenuation of acetic acid formation.

It has been found that, after enhancement, in particular  
20 overexpression of at least one or more of the genes  
selected from the group *iclR* and *fadR*, microorganisms of  
the Enterobacteriaceae family produce L-amino acids, in  
particular L-threonine, in improved manner.

The nucleotide sequences of the genes of *Escherichia coli*  
25 are part of the prior art and may also be obtained by the  
genome sequence of *Escherichia coli* published by Blattner  
et al. (Science 277: 1453-1462 (1997)).

*iclR* gene:

Description: Regulator of central intermediate  
30 metabolism, repressor of *aceBAK* operon  
(*IclR*)

Reference: Sunnarborg et al.; Journal of Bacteriology  
172(5): 2642-2649 (1990); Cortay et al.;  
EMBO Journal 10(3): 675-679 (1991)

35 Accession no.: AE000475

## fadR gene:

Description: Regulator of fatty acid and acetate metabolism (FadR)

Reference: DiRusso; Nucleic Acids Research 16 (16):  
5 7995-8009 (1988); Raman et al.; Journal of  
Biological Chemistry 272(49): 30645-30650  
(1997)

Accession no.: AE000217

Alternative gene names: dec, ole, thdB

- 10 The nucleic acid sequences may be obtained from the  
databases of the National Center for Biotechnology  
Information (NCBI), the National Library of Medicine  
(Bethesda, MD, USA), the nucleotide sequence database of  
the European Molecular Biology Laboratories (EMBL,  
15 Heidelberg, Germany or Cambridge, UK) or the DNA Data Bank  
of Japan (DDBJ, Mishima, Japan).

- The genes described in the stated references may be used  
according to the invention. Alleles of the genes arising  
from the degeneracy of the genetic code or from  
20 functionally neutral sense mutations may also be used.

Enhancement may be achieved, for example, by increasing  
gene expression or the catalytic properties of the  
proteins. Both measures may optionally be combined.

- Overexpression may be achieved by increasing the copy  
25 number of the corresponding genes or by mutating the  
promoter and regulation region or the ribosome-binding site  
located upstream from the structural gene. Expression  
cassettes incorporated upstream from the structural gene  
act in the same manner. It is additionally possible to  
30 increase expression during fermentative L-threonine  
production by means of inducible promoters. Expression is  
also improved by measures to extend the lifetime of the  
mRNA. Enzyme activity is moreover enhanced by preventing  
degradation of the enzyme protein. The genes or gene

constructs may either be present in plasmids in a variable copy number or be integrated in the chromosome and amplified. Alternatively, overexpression of the genes concerned may also be achieved by modifying the composition  
5 of the media and culture conditions.

The person skilled in the art will find guidance in this connection inter alia in Chang and Cohen (Journal of Bacteriology 134: 1141-1156 (1978)), in Hartley and Gregori (Gene 13: 347-353 (1981)), in Amann and Brosius (Gene 40:  
10 183-190 (1985)), in de Broer et al. (Proceedings of the National Academy of Sciences of the United States of America 80: 21-25 (1983)), in LaVallie et al. (BIO/TECHNOLOGY 11: 187-193 (1993)), in PCT/US97/13359, in Llosa et al. (Plasmid 26: 222-224 (1991)), in Quandt and  
15 Klipp (Gene 80:161-169 (1989)), in Hamilton et al. (Journal of Bacteriology 171: 4617-4622 (1989)), in Jensen and Hammer (Biotechnology and Bioengineering 58: 191-195 (1998)) and in known textbooks of genetics and molecular biology.

20 Plasmid vectors replicable in Enterobacteriaceae, such as for example cloning vectors derived from pACYC184 (Bartolomé et al.; Gene 102: 75-78 (1991)), pTrc99A (Amann et al.; Gene 69: 301-315 (1988)) or pSC101 derivatives (Vocke and Bastia; Proceedings of the National Academy of  
25 Sciences of the United States of America 80 (21): 6557-6561 (1983)), may be used. A strain transformed with a plasmid vector may be used in a process according to the invention, wherein the plasmid vector bears at least one or more of the genes selected from the group *iclR* and *fadR*, or  
30 nucleotide sequences which code therefor.

It is also possible to introduce mutations which affect expression of the particular genes into various strains by sequence exchange (Hamilton et al.; Journal of Bacteriology 171: 4617-4622 (1989)), conjugation or transduction.



It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, with strains of the Enterobacteriaceae family, in addition to enhancing one or more of the genes selected from the group *iclR* and *fadR*,  
5 to enhance one or more enzymes of the known threonine biosynthetic pathway or enzymes of anaplerotic metabolism or enzymes for the production of reduced nicotinamide adenine dinucleotide phosphate or enzymes of glycolysis or PTS enzymes or enzymes of sulfur metabolism.

10 It is, for example, possible simultaneously to enhance, in particular overexpress, one or more of the genes selected from the group

- the *thrABC* operon, which codes for aspartate kinase, homoserine dehydrogenase, homoserine kinase and  
15 threonine synthase (US-A-4,278,765),
- the *pyc* gene of *Corynebacterium glutamicum*, which codes for pyruvate carboxylase (WO 99/18228),
- the *pps* gene, which codes for phosphoenolpyruvate synthase (Molecular and General Genetics 231(2): 332-336  
20 (1992)),
- the *ppc* gene, which codes for phosphoenolpyruvate carboxylase (Gene 31: 279-283 (1984)),
- the *pntA* and *pntB* genes, which code for transhydrogenase (European Journal of Biochemistry 158: 647-653 (1986)),
- 25 • the *rhtB* gene, which imparts homoserine resistance (EP-A-0 994 190),
- the *mgo* gene, which codes for malate:quinone oxidoreductase (Journal of Bacteriology 182: 6892-6899 (2000)),
- 30 • the *rhtC* gene, which imparts threonine resistance (EP-A-1 013 765),

- the thrE gene of Corynebacterium glutamicum, which codes for the threonine export protein (EP-A-1 085 091)
- the gdhA gene, which codes for glutamate dehydrogenase (Nucleic Acids Research 11: 5257-5266 (1983); Gene 23: 199-209 (1983)),
- the hns gene, which codes for the DNA-binding protein HLP-II (Molecular and General Genetics 212: 199-202 (1988)),
- the pgm gene, which codes for phosphoglucomutase (Journal of Bacteriology 176: 5847-5851 (1994)),
- the fba gene, which codes for fructose biphosphate aldolase (Biochemical Journal 257: 529-534 (1989)),
- the ptsH gene of the ptsHICrr operon which codes for the phosphohistidine protein hexose phosphotransferase of the phosphotransferase system, PTS (Journal of Biological Chemistry 262: 16241-16253 (1987)),
- the ptsI gene of the ptsHICrr operon which codes for enzyme I of the phosphotransferase system, PTS (Journal of Biological Chemistry 262: 16241-16253 (1987)),
- the crr gene of the ptsHICrr operon which codes for the glucose-specific IIA component of the phosphotransferase system, PTS. (Journal of Biological Chemistry 262: 16241-16253 (1987)),
- the ptsG gene, which codes for the glucose-specific IIBC component (Journal of Biological Chemistry 261: 16398-16403 (1986)),
- the lrp gene, which codes for the regulator of the leucine regulon (Journal of Biological Chemistry 266: 10768-10774 (1991)),

- the mopB gene, which codes for the 10 kD chaperone (Journal of Biological Chemistry 261: 12414-12419 (1986)), which is also known by the name groES,
- 5 • the ahpC gene of the ahpCF operon which codes for the small subunit of alkyl hydroperoxide reductase (Proceedings of the National Academy of Sciences of the United States of America 92: 7617-7621 (1995)),
- 10 • the ahpF gene of the ahpCF operon which codes for the large subunit of alkyl hydroperoxide reductase (Proceedings of the National Academy of Sciences of the United States of America 92: 7617-7621 (1995)),
- the cysK gene, which codes for cysteine synthase A (Journal of Bacteriology 170: 3150-3157 (1988)),
- 15 • the cysB gene, which codes for the regulator of the cys regulon (Journal of Biological Chemistry 262: 5999-6005 (1987)),
- 20 • the cysJ gene of the cysJIH operon which codes for the flavoprotein of NADPH-sulfite reductase (Journal of Biological Chemistry 264: 15796-15808 (1989), Journal of Biological Chemistry 264: 15726-15737 (1989)),
- the cysI gene of the cysJIH operon which codes for the haemoprotein of NADPH-sulfite reductase (Journal of Biological Chemistry 264: 15796-15808 (1989), Journal of Biological Chemistry 264: 15726-15737 (1989)),
- 25 • the cysH gene of the cysJIH operon which codes for adenylyl sulfate reductase (Journal of Biological Chemistry 264: 15796-15808 (1989), Journal of Biological Chemistry 264: 15726-15737 (1989)),
- 30 • the phoB gene of the phoBR operon which codes for the positive regulator PhoB of the pho regulon (Journal of Molecular Biology 190 (1): 37-44 (1986)),

- the *phoR* gene of the *phoBR* operon which codes for the sensor protein of the *pho* regulon (Journal of Molecular Biology 192 (3): 549-556 (1986)),
- 5 • the *phoE* gene, which codes for protein E of the outer cell membrane (Journal of Molecular Biology 163 (4): 513-532 (1983)),
- the *malE* gene, which codes for the periplasmatic binding protein of maltose transport (Journal of Biological Chemistry 259 (16): 10606-10613 (1984)),
- 10 • the *pykF* gene, which codes for fructose-stimulated pyruvate kinase I (Journal of Bacteriology 177 (19): 5719-5722 (1995)),
- the *pfkB* gene, which codes for 6-phosphofructokinase II (Gene 28 (3): 337-342 (1984)),
- 15 • the *talB* gene, which codes for transaldolase B (Journal of Bacteriology 177 (20): 5930-5936 (1995)),
- the *rseA* gene of the *rseABC* operon which codes for a membrane protein with anti- $\sigma^E$  activity (Molecular Microbiology 24 (2): 355-371 (1997)),
- 20 • the *rseC* gene of the *rseABC* operon which codes for a global regulator of the  $\sigma^E$  factor (Molecular Microbiology 24 (2): 355-371 (1997)),
- the *sodA* gene, which codes for superoxide dismutase (Journal of Bacteriology 155 (3): 1078-1087 (1983)),
- 25 • the *sucA* gene of the *sucABCD* operon which codes for the decarboxylase subunit of 2-ketoglutarate dehydrogenase (European Journal of Biochemistry 141 (2): 351-359 (1984)),
- the *sucB* gene of the *sucABCD* operon which codes for the dihydrolipoyltranssuccinase E2 subunit of
- 30

2-ketoglutarate dehydrogenase (European Journal of Biochemistry 141 (2): 361-374 (1984)),

- the *sucC* gene of the *sucABCD* operon which codes for the  $\beta$ -subunit of succinyl-CoA synthetase (Biochemistry 24 (22): 6245-6252 (1985)) and
- the *sucD* gene of the *sucABCD* operon which codes for the  $\alpha$ -subunit of succinyl-CoA synthetase (Biochemistry 24 (22): 6245-6252 (1985)).

It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, in addition to enhancing one or more of the genes selected from the group *iclR* and *fadR*, to attenuate, in particular suppress or reduce the expression of, one or more genes selected from the group

- the *tdh* gene, which codes for threonine dehydrogenase (Journal of Bacteriology 169: 4716-4721 (1987)),
- the *mdh* gene, which codes for malate dehydrogenase (E.C. 1.1.1.37) (Archives in Microbiology 149: 36-42 (1987)),
- the *yfjA* gene product of the open reading frame (orf) (accession number AAC77180 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA)),
- the *ytfP* gene product of the open reading frame (orf) (accession number AAC77179 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA)),
- the *pckA* gene, which codes for the enzyme phosphoenolpyruvate carboxykinase (Journal of Bacteriology 172: 7151-7156 (1990)),
- the *poxB* gene, which codes for pyruvate oxidase (Nucleic Acids Research 14 (13): 5449-5460 (1986)),

- the aceA gene, which codes for the enzyme isocitrate lyase (Journal of Bacteriology 170: 4528-4536 (1988)),
- the dgsA gene, which codes for the DgsA regulator of the phosphotransferase system (Bioscience, Biotechnology and Biochemistry 59: 256-251 (1995)), which is also known by the name mlc gene,
- the fruR gene, which codes for the fructose repressor (Molecular and General Genetics 226: 332-336 (1991)), which is also known by the name cra gene,
- 10 • the rpoS gene which codes for the Sigma<sup>38</sup> factor (WO 01/05939), which is also known by the name katF gene,
- the aspA gene, which codes for aspartate ammonium lyase (aspartase) (Nucleic Acids Research 13(6): 2063-2074 (1985)) and
- 15 • the aceB gene, which codes for malate synthase A (Nucleic Acids Research 16(19): 9342 (1988)).

In this connection, the term "attenuation" means reducing or suppressing the intracellular activity of one or more enzymes (proteins) in a microorganism, which enzymes are coded by the corresponding DNA, for example by using a weak promoter or a gene or allele which codes for a corresponding enzyme which has a low activity or inactivates the corresponding enzyme (protein) or gene and optionally by combining these measures.

25 The attenuation measures reduce the activity or concentration of the corresponding protein in general to 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild type protein, or the activity or concentration of the protein in the starting  
30 microorganism.

It may furthermore be advantageous for the production of L-amino acids, in particular L-threonine, in addition to enhancing one or more of the genes selected from the group *iclR* and *fadR*, to suppress unwanted secondary reactions

- 5 (Nakayama: "Breeding of Amino Acid Producing Micro-organisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

- The microorganisms produced according to the invention may  
10 be cultured using the batch process, the fed batch process or repeated fed batch process. A summary of known culture methods is given in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by  
15 Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

- The culture medium to be used must adequately satisfy the requirements of the particular strains. Culture media for various microorganisms are described in "Manual of Methods  
20 for General Bacteriology" from the American Society for Bacteriology (Washington D.C., USA, 1981).

- Carbon sources which may be used include sugars and carbohydrates, such as for example glucose, sucrose, lactose, fructose, maltose, molasses, starch and optionally  
25 cellulose, oils and fats, such as for example soya oil, sunflower oil, peanut oil and coconut oil, fatty acids, such as for example palmitic acid, stearic acid and linoleic acid, alcohols, such as for example glycerol and ethanol, and organic acids, such as for example acetic  
30 acid. These substances may be used individually or as a mixture.

Nitrogen sources which may be used comprise organic compounds containing nitrogen, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor,

soya flour and urea or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate. The nitrogen sources may be used individually or as a mixture.

- 5 Phosphorus sources which may be used are phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding salts containing sodium. The culture medium must additionally contain salts of metals, such as magnesium sulfate or iron sulfate for example,  
10 which are necessary for growth. Finally, essential growth-promoting substances such as amino acids and vitamins may also be used in addition to the above-stated substances. Suitable precursors may furthermore be added to the culture medium. The stated feed substances may be added to the  
15 culture as a single batch or be fed appropriately during culturing.

- Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or ammonia water, or acidic compounds, such as phosphoric acid or sulfuric acid, are used  
20 appropriately to control the pH of the culture. Foaming may be controlled by using antifoaming agents such as fatty acid polyglycol esters for example. Suitable selectively acting substances, such as for example antibiotics, may be added to the medium in order to maintain plasmid stability.  
25 Oxygen or gas mixtures containing oxygen, such as for example air, are introduced into the culture in order to maintain aerobic conditions. The temperature of the culture is normally from 25°C to 45°C and preferably from 30°C to 40°C. The culture is continued until the maximum quantity  
30 of L-amino acids or L-threonine has formed. This aim is normally achieved within 10 to 160 hours.

- Analysis of L-amino acids may be performed by anion exchange chromatography with subsequent ninhydrin derivation, as described in Spackman et al. (Analytical  
35 Chemistry, 30, 1190-1206 (1958)) or it may be performed by



reversed phase HPLC, as described in Lindroth et al.  
(Analytical Chemistry 51: 1167-1174 (1979)).

The purpose of the process according to the invention is  
the fermentative production of L-amino acids, such as for  
5 example L-threonine, L-isoleucine, L-valine, L-methionine,  
L-homoserine and L-lysine, in particular L-threonine.

The present invention is illustrated in greater detail by  
the following practical examples.

The minimal medium (M9) and complete medium (LB) used for  
10 Escherichia coli are described by J.H. Miller (A Short  
Course in Bacterial Genetics (1992), Cold Spring Harbor  
Laboratory Press). Isolation of plasmid DNA from  
Escherichia coli and all restriction, ligation, Klenow and  
alkaline phosphatase treatment techniques were performed in  
15 accordance with Sambrook et al. (Molecular Cloning - A  
Laboratory Manual (1989) Cold Spring Harbor Laboratory  
Press). Unless otherwise stated, transformation of  
Escherichia coli was performed in accordance with Chung et  
al. (Proceedings of the National Academy of Sciences of the  
20 United States of America 86: 2172-2175 (1989)).

The incubation temperature during production of strains and  
transformants is 37°C.

#### Example 1

Production of L-threonine using the fadR gene

25 1a) Construction of pTrc99AfadR expression plasmid

The fadR gene from E. coli K12 is amplified using the  
polymerase chain reaction (PCR) and synthetic  
oligonucleotides. PCR primers are synthesized (MWG Biotech,  
Ebersberg, Germany) on the basis of the nucleotide sequence  
30 of the fadR gene in E. coli K12 MG1655 (accession no.  
AE000217, Blattner et al. (Science 277: 1453-1462 (1997))).

The primer sequences are modified in such a manner that recognition sites for restriction enzymes are obtained. The recognition sequence for XbaI is selected for the fadR3 primer, while the recognition sequence for HindIII is selected for the fadR4 primer, these sequences being indicated by the underlined portions of the nucleotide sequence shown below:

fadR3: 5' - GTCCAACTTTGTCTAGATGAGTTATGG - 3' (SEQ ID no. 1)

fadR4: 5' - GAGGGGTTTGAAGCTTAAACGGAAGGG - 3' (SEQ ID no. 2)

- 10 The chromosomal E. coli K12 MG1655 DNA used for the PCR is isolated using "Qiagen Genomic-tips 100/G" (QIAGEN, Hilden, Germany) in accordance with the manufacturer's instructions. An approx. 800 bp DNA fragment can be amplified with the specific primers under standard PCR conditions (Innis et al. (1990) PCR Protocols. A Guide to Methods and Applications, Academic Press) using Pfu DNA polymerase (Promega Corporation, Madison, USA). The PCR product is ligated in accordance with the manufacturer's instructions with the vector pCR-Blunt II-TOPO (Zero Blunt TOPO PCR Cloning Kit, Invitrogen, Groningen, Netherlands) and transformed into E. coli strain TOP10. Plasmid-bearing cells are selected on LB agar which has been combined with 50 µg/ml of kanamycin. After isolation of the plasmid DNA, the vector pCR-Blunt II-TOPO-fadR is cleaved with the restriction enzymes HindIII and XbaI and, after separation, the fadR fragment is isolated in 0.8% agarose gel using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). The vector pTrc99A (Pharmacia Biotech, Uppsala, Sweden) is cleaved with the enzymes HindIII and XbaI and ligated with the isolated fadR fragment. The E. coli strain XL1-Blue MRF' (Stratagene, La Jolla, USA) is transformed with the ligation batch and plasmid-bearing cells are selected on LB agar, which has been combined with 50 µg/ml of ampicillin. Success of the cloning can be verified after plasmid DNA isolation by performing a test cleavage with the enzymes

AccI, HincII and SspI. The plasmid is designated pTrc99AfadR (Figure 1).

1b) Production of L-threonine with strain  
MG442/pTrc99AfadR

- 5 The L-threonine-producing E. coli strain MG442 is described in patent US-A-4,278,765 and has been deposited as CMIM B-1628 at the Russian National Collection of Industrial Microorganisms (VKPM, Moscow, Russia).

Strain MG442 is transformed with the expression plasmid  
10 pTrc99AfadR described in Example 1a and the vector pTrc99A and plasmid-bearing cells are selected on LB agar with 50 µg/ml of ampicillin. In this manner, strains MG442/pTrc99AfadR and MG442/pTrc99A are obtained. Selected individual colonies are then further multiplied on minimal  
15 medium of the following composition: 3.5 g/l Na<sub>2</sub>HPO<sub>4</sub>\*2H<sub>2</sub>O, 1.5 g/l KH<sub>2</sub>PO<sub>4</sub>, 1 g/l NH<sub>4</sub>Cl, 0.1 g/l MgSO<sub>4</sub>\*7H<sub>2</sub>O, 2 g/l glucose, 20 g/l agar, 50 mg/l ampicillin. Formation of L-threonine is verified in 10 ml batch cultures in 100 ml Erlenmeyer flasks. Said culture is inoculated with 10 ml of  
20 preculture medium of the following composition: 2 g/l yeast extract, 10 g/l (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1 g/l KH<sub>2</sub>PO<sub>4</sub>, 0.5 g/l MgSO<sub>4</sub>\*7H<sub>2</sub>O, 15 g/l CaCO<sub>3</sub>, 20 g/l glucose, 50 mg/l ampicillin and incubated for 16 hours at 37°C and 180 rpm in an ESR incubator from Kühner AG (Birsfelden, Switzerland).

25 250 µl portions of this preculture are transferred into 10 ml of production medium (25 g/l (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2 g/l KH<sub>2</sub>PO<sub>4</sub>, 1 g/l MgSO<sub>4</sub>\*7H<sub>2</sub>O, 0.03 g/l FeSO<sub>4</sub>\*7H<sub>2</sub>O, 0.018 g/l MnSO<sub>4</sub>\*1H<sub>2</sub>O, 30 g/l CaCO<sub>3</sub>, 20 g/l glucose, 50 mg/l ampicillin) and incubated for 48 hours at 37°C. Formation of L-threonine by  
30 the starting strain MG442 is verified in the same manner, but without the addition of ampicillin to the medium. After incubation, the optical density (OD) of the culture suspension is determined at a measurement wavelength of

660 nm using an LP2W photometer from the company Dr. Lange (Düsseldorf, Germany).

The concentration of L-threonine formed is then determined in the sterile-filtered culture supernatant using an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column reaction with ninhydrin detection.

Table 1 shows the result of the test.

Table 1

Strain	OD (660 nm)	L-threonine g/l
MG442	5.6	1.4
MG442/pTrc99A	3.8	1.3
MG442/pTrc99AfadR	4.0	1.6

10

### Example 2

Production of L-threonine using the *iclR* gene

#### 2a) Construction of pTrc99A*iclR* expression plasmid

The *iclR* gene from *E. coli* K12 is amplified using the polymerase chain reaction (PCR) and synthetic oligonucleotides. PCR primers are synthesized (MWG Biotech, Ebersberg, Germany) on the basis of the nucleotide sequence of the *iclR* gene in *E. coli* K12 MG1655 (accession no. AE000475, Blattner et al. (Science 277: 1453-1462 (1997))). The primer sequences are modified in such a manner that recognition sites for restriction enzymes are obtained. The recognition sequence for *Xba*I is selected for the *iclR*3 primer, while the recognition sequence for

HindIII is selected for the iclR4 primer, these sequences being indicated by the underlined portions of the nucleotide sequence shown below:

iclR3: 5' - CAGTTCAGTATCTAGAGCATGAGCTAAC - 3'  
5 (SEQ ID no. 3)

iclR4: 5' - GGTATGATGGGCAGAAAGCTTGCCTCTGC - 3'  
(SEQ ID no. 4)

The chromosomal E. coli K12 MG1655 DNA used for the PCR is isolated using "Qiagen Genomic-tips 100/G" (QIAGEN, Hilden, Germany) in accordance with the manufacturer's instructions. An approx. 950 bp DNA fragment can be amplified with the specific primers under standard PCR conditions (Innis et al. (1990) PCR Protocols. A Guide to Methods and Applications, Academic Press) using Pfu DNA polymerase (Promega Corporation, Madison, USA). The PCR product is ligated in accordance with the manufacturer's instructions with the vector pCR-Blunt II-TOPO (Zero Blunt TOPO PCR Cloning Kit, Invitrogen, Groningen, Netherlands) and transformed into E. coli strain TOP10.

Plasmid-bearing cells are selected on LB agar which has been combined with 50 µg/ml of kanamycin. After isolation of the plasmid DNA, the vector pCR-Blunt II-TOPO-iclR is cleaved with the restriction enzymes HindIII and XbaI and, after separation, the iclR fragment is isolated in 0.8% agarose gel using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). The vector pTrc99A (Pharmacia Biotech, Uppsala, Sweden) is cleaved with the enzymes HindIII and XbaI and ligated with the isolated iclR fragment. The E. coli strain XL1-Blue MRF' (Stratagene, La Jolla, USA) is transformed with the ligation batch and plasmid-bearing cells are selected on LB agar, which has been combined with 50 µg/ml of ampicillin. Success of the cloning can be verified after plasmid DNA isolation by performing a test

cleavage with the enzymes BstEII, MluI and RsaI. The plasmid is designated pTrc99AiclR (Figure 2).

2b) Production of L-threonine with strain  
MG442/pTrc99AiclR

- 5 The L-threonine-producing E. coli strain MG442 is described in patent US-A-4,278,765 and has been deposited as CMIM B-1628 at the Russian National Collection of Industrial Microorganisms (VKPM, Moscow, Russia).

- Strain MG442 is transformed with the expression plasmid
- 10 pTrc99AiclR described in Example 2a and the vector pTrc99A and plasmid-bearing cells are selected on LB agar with 50 µg/ml of ampicillin. In this manner, strains MG442/pTrc99AiclR and MG442/pTrc99A are obtained. Selected individual colonies are then further multiplied on minimal
- 15 medium of the following composition: 3.5 g/l  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , 1.5 g/l  $\text{KH}_2\text{PO}_4$ , 1 g/l  $\text{NH}_4\text{Cl}$ , 0.1 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2 g/l glucose, 20 g/l agar, 50 mg/l ampicillin. Formation of L-threonine is verified in 10 ml batch cultures in 100 ml Erlenmeyer flasks. Said culture is inoculated with 10 ml of
- 20 preculture medium of the following composition: 2 g/l yeast extract, 10 g/l  $(\text{NH}_4)_2\text{SO}_4$ , 1 g/l  $\text{KH}_2\text{PO}_4$ , 0.5 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 15 g/l  $\text{CaCO}_3$ , 20 g/l glucose, 50 mg/l ampicillin and incubated for 16 hours at 37°C and 180 rpm in an ESR incubator from Kühner AG (Birsfelden, Switzerland).
- 25 250 µl portions of this preculture are transferred into 10 ml of production medium (25 g/l  $(\text{NH}_4)_2\text{SO}_4$ , 2 g/l  $\text{KH}_2\text{PO}_4$ , 1 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.03 g/l  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.018 g/l  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 30 g/l  $\text{CaCO}_3$ , 20 g/l glucose, 50 mg/l ampicillin) and incubated for 48 hours at 37°C. Formation of L-threonine by
- 30 the starting strain MG442 is verified in the same manner, but without the addition of ampicillin to the medium. After incubation, the optical density (OD) of the culture suspension is determined at a measurement wavelength of

660 nm using an LP2W photometer from the company Dr. Lange (Düsseldorf, Germany).

The concentration of L-threonine formed is then determined in the sterile-filtered culture supernatant using an amino  
5 acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column reaction with ninhydrin detection.

Table 2 shows the result of the test.

Table 2

Strain	OD (660 nm)	L-threonine g/l
MG442	5.6	1.4
MG442/pTrc99A	3.8	1.3
MG442/pTrc99AiclR	5.7	2.8

10

#### Brief Description of the Figures:

Figure 1: Map of plasmid pTrc99AfadR containing the fadR gene

Figure 2: Map of plasmid pTrc99AiclR containing the iclR  
15 gene

The lengths stated should be considered to be approximate. The abbreviations and terms used have the following meaning:

- Amp: ampicillin resistance gene
- 20 • lacI: gene for the trc-promoter repressor protein
- Ptrc: trc-promoter region, IPTG-inducible

- fadR: coding region of the fadR gene
- iclR: coding region of the iclR gene
- 5S: 5S rRNA region
- rrnBT: rRNA terminator region

5 The abbreviations for the restriction enzymes have the following meaning

- AccI: restriction endonuclease from *Acinetobacter calcoaceticus*
- 10 • BstEII: restriction endonuclease from *Bacillus stearothermophilus* ATCC 12980
- HincII: restriction endonuclease from *Haemophilus influenzae* R<sub>c</sub>
- HindIII: restriction endonuclease from *Haemophilus influenzae*
- 15 • MluI: restriction endonuclease from *Micrococcus luteus* IFO 12992
- RsaI: restriction endonuclease from *Rhodopseudomonas sphaeroides*
- SspI: restriction endonuclease from *Sphaerotilus* species  
20 ATCC 13925
- XbaI: restriction endonuclease from *Xanthomonas campestris*



**What is claimed is:**

1. A process for the production of L-amino acids, in particular L-threonine, wherein the following steps are performed:
  - 5 a) fermentation of microorganisms of the Enterobacteriaceae family which produce the desired L-amino acid, in which one or more of the genes, selected from the group *iclR* and *fadR*, or nucleotide sequences coding therefor, are enhanced,  
10 in particular overexpressed,
  - b) accumulation of the desired L-amino acid in the medium or in the cells of the microorganisms and
  - c) isolation of the desired L-amino acid, wherein  
15 constituents of the fermentation broth and/or the biomass in their entirety or fractions thereof (>0 to 100%) optionally remain in the product.
2. A process according to claim 1, wherein  
20 microorganisms are used in which further genes in the biosynthetic pathway of the desired L-amino acid are additionally enhanced.
3. A process according to claim 1, wherein  
microorganisms are used in which the metabolic pathways which reduce the formation of the desired L-amino acid are at least partially suppressed.
- 25 4. A process according to claim 1, wherein expression of the polynucleotide(s) which code(s) for one or more of the genes selected from the group *iclR* and *fadR* is increased.
- 30 5. A process according to claim 1, wherein the regulatory and/or catalytic properties of the

polypeptides (proteins) for which the polynucleotides *iclR* and *fadR* code are improved or increased.

- 5 6. A process according to claim 1, wherein L-amino acids are produced by fermenting microorganisms of the Enterobacteriaceae family in which one or more genes selected from the following group is/are simultaneously enhanced, in particular overexpressed:

- 10 6.1 the *thrABC* operon, which codes for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase,
- 6.2 the *pyc* gene, which codes for pyruvate carboxylase,
- 6.3 the *pps* gene, which codes for phosphoenolpyruvate synthase,
- 15 6.4 the *ppc* gene, which codes for phosphoenolpyruvate carboxylase,
- 6.5 the *pntA* and *pntB* genes, which code for transhydrogenase,
- 20 6.6 the *rhtB* gene, which imparts homoserine resistance,
- 6.7 the *mgo* gene, which codes for malate:quinone oxidoreductase,
- 6.8 the *rhtC* gene, which imparts threonine resistance,
- 25 6.9 the *thrE* gene, which codes for the threonine export protein,
- 6.10 the *gdhA* gene, which codes for glutamate dehydrogenase,

- 6.11 the hns gene, which codes for the DNA-binding protein HLP-II,
- 6.12 the pgm gene, which codes for phosphoglucomutase,
- 5 6.13 the fba gene, which codes for fructose biphosphate aldolase,
- 6.14 the ptsH gene, which codes for phosphohistidine protein hexose phosphotransferase,
- 10 6.15 the ptsI gene, which codes for enzyme I of the phosphotransferase system,
- 6.16 the crr gene, which codes for the glucose-specific IIA component,
- 6.17 the ptsG gene, which codes for the glucose-specific IIBC component,
- 15 6.18 the lrp gene, which codes for the regulator of the leucine regulon,
- 6.19 the mopB gene, which codes for the 10 kD chaperone,
- 20 6.20 the ahpC gene, which codes for the small subunit of alkyl hydroperoxide reductase,
- 6.21 the ahpF gene, which codes for the large subunit of alkyl hydroperoxide reductase,
- 6.22 the cysK gene, which codes for cysteine synthase A,
- 25 6.23 the cysB gene, which codes for the regulator of the cys regulon,
- 6.24 the cysJ gene, which codes for the flavoprotein of NADPH-sulfite reductase,

- 6.25 the cysI gene, which codes for the haemoprotein of NADPH-sulfite reductase,
- 6.26 the cysH gene, which codes for adenylyl sulfate reductase,
- 5 6.27 the phoB gene, which codes for the positive regulator PhoB of the pho regulon,
- 6.28 the phoR gene, which codes for the sensor protein of the pho regulon,
- 10 6.29 the phoE gene, which codes for protein E of the outer cell membrane,
- 6.30 the malE gene, which codes for the periplasmatic binding protein of maltose transport,
- 15 6.31 the pykF gene, which codes for fructose-stimulated pyruvate kinase I,
- 6.32 the pfkB gene, which codes for 6-phosphofructokinase II,
- 6.33 the talB gene, which codes for transaldolase B,
- 20 6.34 the rseA gene, which codes for a membrane protein which acts as a negative regulator on sigmaE activity,
- 6.35 the rseC gene, which codes for a global regulator of the sigmaE factor,
- 25 6.36 the sodA gene, which codes for superoxide dismutase,
- 6.37 the sucA gene, which codes for the decarboxylase subunit of 2-ketoglutarate dehydrogenase,

- 6.38 the sucB gene, which codes for the dihydrolipoyltranssuccinase E2 subunit of 2-ketoglutarate dehydrogenase,
- 5 6.39 the sucC gene, which codes for the  $\beta$ -subunit of succinyl-CoA synthetase and
- 6.40 the sucD gene, which codes for the  $\alpha$ -subunit of succinyl-CoA synthetase.
7. A process according to claim 1, wherein L-amino acids are produced by fermenting microorganisms of the Enterobacteriaceae family in which one or more genes selected from the following group is/are simultaneously attenuated, in particular suppressed, or expression is reduced:
- 10
- 7.1 the tdh gene, which codes for threonine dehydrogenase,
- 15
- 7.2 the mdh gene, which codes for malate dehydrogenase,
- 7.3 the yfjA gene product of the open reading frame (orf),
- 20 7.4 the ytfP gene product of the open reading frame (orf),
- 7.5 the pckA gene, which codes for phosphoenolpyruvate carboxykinase,
- 25 7.6 the poxB gene, which codes for pyruvate oxidase,
- 7.7 the aceA gene, which codes for isocitrate lyase,
- 7.8 the dgsA gene, which codes for the DgsA regulator of the phosphotransferase system,

- 7.9 the fruR gene, which codes for the fructose repressor,
- 7.10 the rpoS gene which codes for the Sigma<sup>38</sup> factor,
- 5 7.11 the aspA gene, which codes for aspartate ammonium lyase (aspartase) and
- 7.12 the aceB gene, which codes for malate synthase A.

Figure 1:

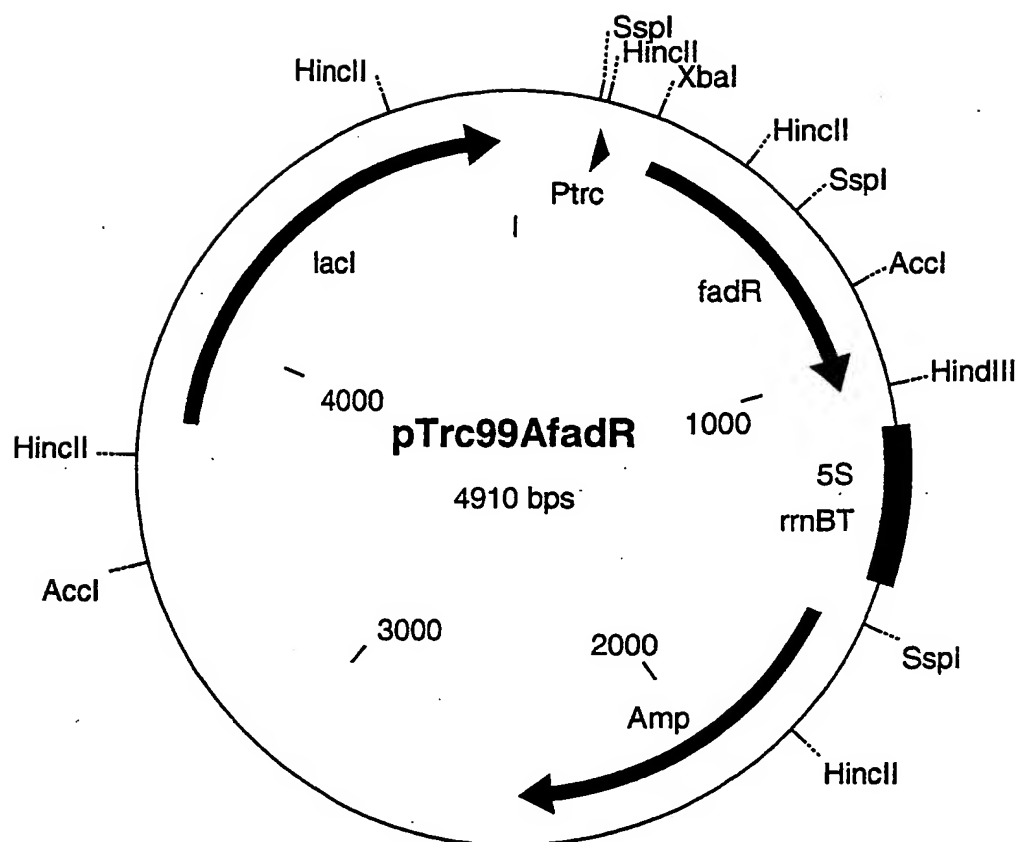
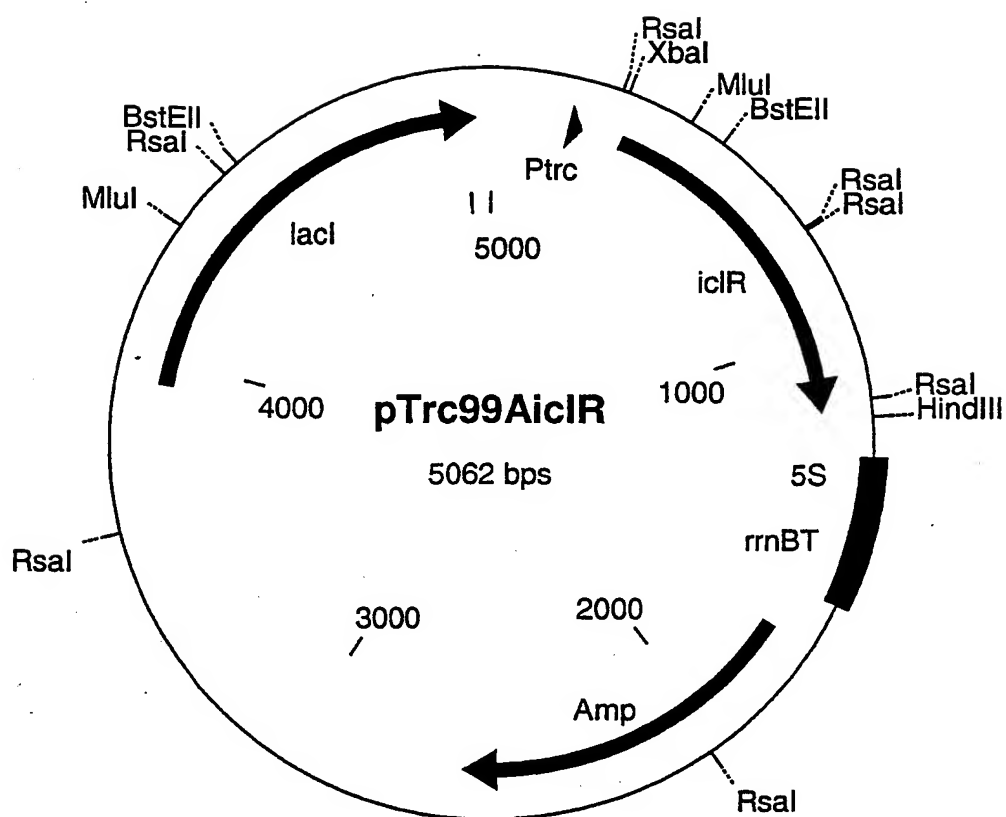


Figure 2:





## SEQUENCE LISTING

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<120> Process for the production of L-amino acids using  
10 strains of the Enterobacteriaceae family which  
contain an enhanced fadR or iclR gene

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28

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(54) Title: A PROCESS FOR THE PRODUCTION OF L-AMINO ACIDS USING STRAINS OF THE ENTEROBACTERIACEAE FAMILY WHICH CONTAIN AN ENHANCED FADR OR ICLR GENE

(57) Abstract: This invention relates to a process for the production of L-amino acids, in particular L-threonine, in which the following steps are performed: a) fermentation of microorganisms of the Enterobacteriaceae family which produce the desired L-amino acid, in which at least one or more of the genes, selected from the group iclR and fadR, or nucleotide sequences coding therefor, are enhanced, in particular overexpressed, b) accumulation of the desired L-amino acid in the medium or in the cells of the bacteria and c) isolation of the desired L-amino acid.

**Signal Application No**

### A. CLASSIFICATION OF SUBJECT MATTER

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**B. FIELDS SEARCHED**

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Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

WPI Data, PAJ, EPO-Internal, BIOSIS, MEDLINE

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

☒ Further documents are listed in the continuation of box C.

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- \*Z\* document member of the same patent family

Date of the actual completion of the international search

11 July 2003

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International Application No

PCT/EP 02/10791

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>GUI L ET AL: "Autoregulation of <i>iclR</i>, the gene encoding the repressor of the glyoxylate bypass operon." JOURNAL OF BACTERIOLOGY. US, vol. 178, no. 1, January 1996 (1996-01), pages 321-324, XP002239531 ISSN: 0021-9193 the whole document</p>	1-7
A	<p>CORTAY J C ET AL: "Regulation of the acetate operon in <i>Escherichia coli</i>: purification and functional characterization of the <i>IclR</i> repressor." THE EMBO JOURNAL. ENGLAND , vol. 10, no. 3, March 1991 (1991-03), pages 675-679, XP002239532 ISSN: 0261-4189 cited in the application the whole document</p>	1-7
A	<p>DONALD L J ET AL: "Mass spectrometric study of the <i>Escherichia coli</i> repressor proteins, <i>IclR</i> and <i>GclR</i>, and their complexes with DNA." PROTEIN SCIENCE: A PUBLICATION OF THE PROTEIN SOCIETY. UNITED STATES JUL 2001, vol. 10, no. 7, July 2001 (2001-07), pages 1370-1380, XP008016713 ISSN: 0961-8368 the whole document</p>	1-7
A	<p>XU Y ET AL: "The <i>FadR</i>.DNA complex. Transcriptional control of fatty acid metabolism in <i>Escherichia coli</i>." THE JOURNAL OF BIOLOGICAL CHEMISTRY. US, vol. 276, no. 20, 18 May 2001 (2001-05-18), pages 17373-17379, XP002247351 ISSN: 0021-9258 the whole document</p>	1-7
A	<p>FARMER W R ET AL: "Reduction of aerobic acetate production by <i>Escherichia coli</i>." APPLIED AND ENVIRONMENTAL MICROBIOLOGY. US, vol. 63, no. 8, August 1997 (1997-08), pages 3205-3210, XP002247352 ISSN: 0099-2240 the whole document</p>	1-7

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## INTERNATIONAL SEARCH REPORT

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>DIRUSSO C C ET AL: "Fatty acyl-CoA binding domain of the transcription factor FadR. Characterization by deletion, affinity labeling, and isothermal titration calorimetry." THE JOURNAL OF BIOLOGICAL CHEMISTRY. US, vol. 273, no. 50, 11 December 1998 (1998-12-11), pages 33652-33659, XP002247353 ISSN: 0021-9258 the whole document</p>	1-7
A	<p>LANDGRAF J R ET AL: "THE ROLE OF H-NS IN ONE CARBON METABOLISM" BIOCHIMIE, MASSON, PARIS, FR, vol. 76, no. 10/11, 1994, pages 1063-1070, XP008014239 ISSN: 0300-9084 the whole document</p>	6

# INTERNATIONAL SEARCH REPORT

national application No.  
PCT/EP 02/10791

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-7 partial

Process for the production of L-amino acids by fermenting a microorganism of the Enterobacteriaceae family in which the IclR gene is enhanced (overexpressed). Said process in which further genes of the biosynthetic pathway for the desired L-amino acid are additionally enhanced. Said process in which metabolic pathways which reduce the formation of the desired amino acid are at least partially suppressed. Said process in which the regulatory and/or catalytic properties of the by the IclR gene encoded protein are improved or increased. Said process in which one or more genes selected from the following group are additionally enhanced: thrABC, pyc, pps, ppc, pntA, rhtb, mgo, rhtC, thrE, gdhA, hns, pgm, fba, ptsh, ptsI, crr, ptsG, lrp, mopB, ahpC, ahpF, cysK, cysB, cysJ, cysI, cysH, phoB, phoR, phoE, malE, pykF, pfkB, talB, rseC, sodA, sucA, sucB, sucC, and/or sucD. Said process in which one or more genes selected from the following group is/are simultaneously attenuated or expression is reduced: tdh, mdh, yfjA, ytfP, pckA, poxB, aceA, dgsA, fruR, rpoS, aspA, and/or aceB.

2. Claims: 1-7 partial

As for subject 1, but now the fadR gene is enhanced (overexpressed).



## INTERNATIONAL SEARCH REPORT

Information on patent family members

Initial Application No.

PCT/EP 02/10791

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
US 4347318	A	31-08-1982	JP 1029559 B	12-06-1989
			JP 1552063 C	23-03-1990
			JP 55131397 A	13-10-1980
			DE 3012921 A1	23-10-1980
			FR 2453216 A1	31-10-1980
			GB 2049670 A ,B	31-12-1980